

**Annual Progress Report**  
USEPA STAR Grant RD832095  
“Pulmonary Biomarkers Based on Alterations in Protein Expression  
Following Exposure to Arsenic”  
R. Clark Lantz, PI

(1) Brief statement covering work status

The original objectives and goals of the grant are given below. There have been no changes from the original application. Details of the results to date are given in Section (5) below.

1. Evaluate the effects of chronic exposure to arsenic on expression of proteins using an *in vitro* human bronchial epithelial cell line.
2. Evaluate the effects of chronic exposure to arsenic on expression of proteins in lung lining fluid and cells and in pulmonary epithelium using an *in vivo* animal model.
3. Using proteins identified in Objectives 1 and 2, determine the alterations of these proteins in induced sputum in human populations.

Briefly, we have continued our research into the effects of arsenic on cell migration and wound healing in human bronchial epithelial cells exposed to arsenite or monomethylarsonic acid (MMA(III)). Exposure to arsenic affects epithelial barrier function and cell signaling (Objective 1). This year we have extended our proteomics analysis of lung lining fluid from chronically exposed mice to also examine proteins isolated from airway epithelial cells. We have also tested the effects of arsenic on airway epithelial repair processes in mice. (Objective 2). Additional, putative biomarkers have been analyzed in human induced-sputum and serum samples (Objective 3).

(2) Key personnel

There is no change in the major personnel

(3) Expenditures to date

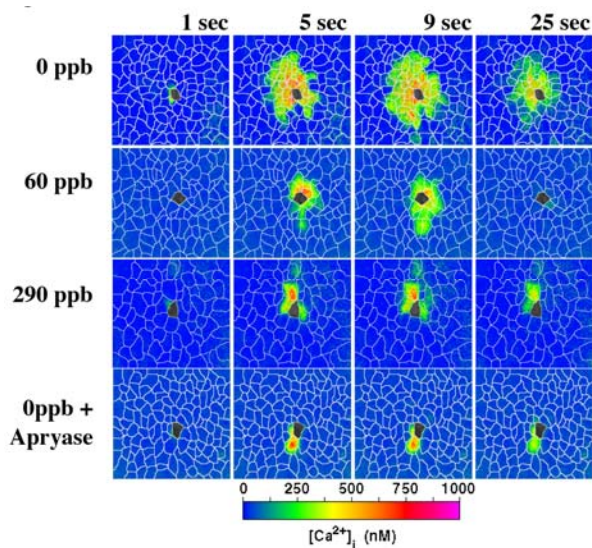
Expenditures to date are approximately 95% of the total grant funds available. Costs are close to what was originally proposed in the grant. We are in a one year no-cost extension.

(4) Quality assurance

Quality assurance is being addressed as outlined in the original QA Statement. Dr. Lantz is responsible for overall compliance. SOPs are in place for all procedures. Instrumentation calibration is routinely performed. Sample collection is performed using standard techniques to preserve the integrity of the samples, while not compromising the identity of the individuals.

## (5) Results to date

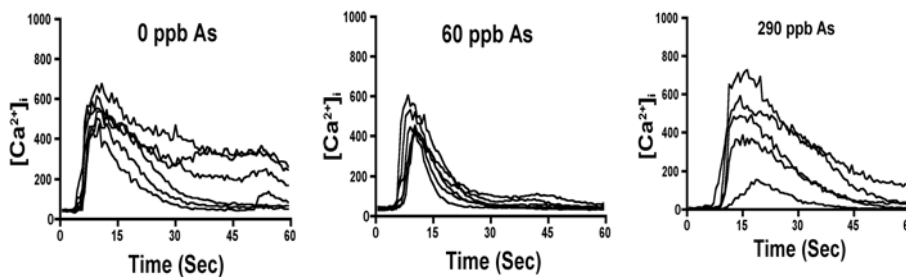
We have developed a unique **integrative partnership including cellular, model organism and human studies in order to identify potential pulmonary biomarkers of arsenic exposure**. Dr. Boitano works with transepithelial resistance and second messenger signaling in airway epithelial cell cultures. Dr. Lantz evaluates arsenic exposure on rodent lungs following chronic arsenic exposure using proteomic and morphological analysis. Dr. Burgess has experience with evaluation of human sputum biomarkers. **Success in our collaborative approach has been documented in our publications (See below)**



### ***In vitro* assays to evaluate arsenic exposure on lung tissue.**

We have continued examining alterations in calcium signaling in human bronchial epithelial (16HBE14o-) cells following arsenic exposure. We have evaluated the effects of 24 hr exposure of arsenic on purnergic dependent  $\text{Ca}^{2+}$  signaling in the airway using wound models and direct ATP application. We have found that arsenic limits the initial  $\text{Ca}^{2+}$  signaling from the site of a scrape wound and that this signaling is ATP dependent. We have further quantified ATP signaling in wound response using a localized wound model (right). In these experiments, we

break open a single cell and evaluate  $\text{Ca}^{2+}$  signaling. Again, the intercellular  $\text{Ca}^{2+}$  signal is reduced after arsenic exposure (24 hr) and eliminated by apyrase treatment (demonstrating a purnergic dependence). In addition, there is a dose dependent reduction in the amount of cells participating in the  $\text{Ca}^{2+}$  wave after mechanical wounding and in response to increasing arsenic. Further quantification of cell immediately adjacent to the wound elucidates subtle changes in  $\text{Ca}^{2+}$  signaling.



In typical experiments (left) the 60ppb samples recover much faster, the 290 ppb samples display a varied response. In all experiments the

0 and 60 ppb samples display a similar  $[\text{Ca}^{2+}]_i$  peak change, however the 290 ppb samples are reduced in peak  $[\text{Ca}^{2+}]_i$ . Additionally, although almost all adjacent cells respond with a  $[\text{Ca}^{2+}]_i$  change in the 0 and 60 ppb samples, the 290ppb samples are reduced to ~75%. Upon alignment of  $\text{Ca}^{2+}$  peaks in the adjacent cells, we can

additionally measure the rate of recovery of  $[Ca^{2+}]_i$  to resting levels. The half-time to recovery for the 60 ppb sample is 1.7 times as fast as that observed in the 0ppb and 290 ppb samples. Effectively, this translates to a reduction of  $Ca^{2+}$  signal in the adjacent cells. In conclusion, there is a loss of a wound-induced  $Ca^{2+}$  signaling response in cells treated with arsenic. We are currently working on elucidating mechanisms that underlie arsenic/ $Ca^{2+}$ /wound repair.

### ***In vivo* animal responses to arsenic exposure**

We have continued to expand our analysis of altered protein expression in the lungs mice exposed to arsenic in their drinking water for up to 4 weeks. In the past we have analyzed soluble proteins in the lung lining fluid. In the past year we have collected samples from the airway epithelial cells, specifically. In order to isolate proteins from the airway epithelium, we have used a selective digestion technique. Briefly, lungs from mice are infused with warm agar to inflate the distal lung. After allowing the agar to harden, the airway epithelial cells are digested using an enzyme mixture. Isolation of epithelial proteins is verified by analyzing for the presence of an airway specific cytochrome P-450. Proteins were identified using multidimensional protein identification technology (MUDPIT) (N=3) coupled with mass spectrometry (MS). Spectra were searched against a FASTA format protein database using the SEQUEST program to provide unambiguous protein identification. Confidence in the protein identification was performed using Scaffold and the confidence was set to greater than 90%. Using these criteria a total of 221 proteins were identified in either controls and/or treated (50 ppb) samples. Using percent spectra as a semi-quantitative index, we have found that 126 proteins were downregulated, 50 were up regulated and 45 were unchanged (less than 2 fold change). Preliminary analysis indicates the most likely cell functions and disease states that are associated with the altered proteins are associated with wound repair and cytoskeleton (MetaCore). These are pathways that we have previously identified as being affected by the chronic arsenic exposure..

We have previously shown that arsenic inhibits ‘wound repair’ in our in vitro model. In order to verify this in an in vivo model, we have utilized a model that has previously been used to determine the effects of cigarette smoke on airway epithelial repair. We have used a naphthalene model of distal airway injury to determine whether arsenic affects airway repair mechanisms. Animals were exposed to 50 ppb arsenic in their water for 4 weeks. Appropriate controls received filtered water. After 4 weeks, animals were exposed to ip injection of naphthalene. This compound is selectively metabolized in the distal lung Clara cells, where one of the metabolites is toxic and kills the cells. With naphthalene alone, the injured epithelium is repaired in approximately 2 weeks. We have therefore examined the distal airways of mice that have been given arsenic alone, naphthalene alone or have had the combined exposures. In order to evaluate the effects, lungs from mice were fixed and embedded in plastic. Areas containing terminal bronchioles were selected. Qualitative examination of the distal airways revealed that after lungs from arsenic alone or from lungs 2 weeks after naphthalene exposure looked similar to control animals. However, animals that had been on arsenic for 4 weeks and had then been administered naphthalene showed altered distal airways. The numbers of Clara cells in this group appeared to be greatly reduced. In some locations, the

epithelium was squamous rather than columnar and in other locations there appeared to be a complete absence of the airway epithelium. Therefore, it appears that *in vivo*, arsenic at 50 ppb is also inhibiting the ability of the airway epithelium to adequately repair damage. We are currently using morphometric techniques to quantify our subjective observations.

### Human population studies

Based on the results from both the *in vitro* and *in vivo* experiments, we have previously analyzed arsenic-induced changes in MMP-9 and RAGE in induced sputum in human populations. Samples were collected from 56 individuals living in Ajo, Arizona (tap water arsenic ~ 20 ppb) and from Tucson, Arizona (tap water arsenic ~ 5 ppb). First morning void urine was also collected and arsenic speciation analysis was performed. ELISA methods were used to determine protein levels of MMP-2, MMP-9, TIMP-1 and RAGE. We have previously shown that total urinary inorganic arsenic was positively correlated with log MMP-9/TIMP-1 ratio in sputum (Pearson's  $\rho = 0.351$ ,  $p = 0.009$ ) and negatively correlated with the log of sputum levels of TIMP-1. In addition, urinary inorganic arsenic was also negatively correlated with levels of sputum RAGE ( $p=0.036$ ). We have continued our population studies by including 382 subjects from Mexico. We have collected urine and speciated the arsenic species. This has been correlated with MMP-9 and TIMP-1 levels in serum collected from the same individuals. The mean total urinary arsenic in this population was 67 ppb. We have found that serum MMP-9 levels was positively correlated with urinary As+5 levels and was borderline significant with total urinary arsenic.

	N	Minimum	Maximum	Mean	Std. Deviation
as_iii_	482	.11	718.29	26.5985	67.15220
dma_v_	484	.36	762.10	23.9998	50.15593
mma_v_	482	.15	261.44	4.7376	15.62781
as_v_	332	.01	95.76	1.7138	6.99997
total_ur Total_urine_As	484	1.55	1121.00	67.1758	101.61333
TIMP1_bd serum conc TIMP-1 ng/ml	382	88.235	433.580	193.53649	45.780980
MMP9_bd serum conc MMP-9 ng/ml	382	15.780	1503.050	373.35118	244.459284

In summary, we have demonstrated that low-level arsenic exposure is associated with alterations in biomarkers associated with lung remodeling. We have shown an association of MMP-9/TIMP-1 and RAGE with arsenic exposure in a US population with relatively low exposure levels of arsenic in drinking water. We have also shown a correlation of serum MMP-9 with arsenic in a higher exposed population from Mexico. Wound healing and pulmonary airway remodeling can occur as a consequence of cigarette smoking and the development of COPD. That MMP-9, RAGE and AAT have been implicated in chronic disease and are therefore presumably involved in lung injury, and that both are affected by arsenic in our test systems shows the power of using multiple test systems to identify affected pathways and to identify potential biomarkers. Combining the use of *in vitro*, animal *in vivo* and human test systems provides us with unique strengths for identifying the effects of pulmonary toxicants.

(6) Planned activity

We will continue these activities. For the *in vitro* experiments, we are continuing to analyze the effect of the arsenic on epithelial barrier function and are assessing whether removal of the arsenic can restore normal function. We will also continue to investigate calcium signaling as a site of action. For *in vivo*, we will continue to analyze the protein set that we have identified from airway epithelium. We will also acquire quantitative data on morphological changes related to wound repair in our animal model. Finally, our human studies will also continue, with the inclusion of samples collected from a Mexican population that is being exposed to 50 ppb and higher arsenic levels in their drinking water. We are also continuing to analyze serum levels of MMP-9 as an indicator of arsenic exposure and effect.

(7) Publications and presentations

Josyula, Arun B., Gerald S. Poplin, Margaret Kurzius-Spencer, Hannah E. McClellen, Michael J. Kopplin, Stefan Sturup, R. Clark **Lantz**, Jefferey L. **Burgess**. Environmental arsenic exposure and sputum metalloproteinase concentrations. Environ. Research 102:283-290, 2006.  
DOI:10.1016/j.envres.2006.01.003

**Lantz**, R. Clark Lantz and Allison M. Hays. Role of oxidative stress in arsenic-induced toxicity. Drug Metabolism Reviews 38:791-804, 2006.  
DOI:10.1080/03602530600980108

**Lantz**, R. Clark, Brandon J. Lynch, Scott **Boitano**, Gerald S. Poplin, Sally Littau , George Tsaprailis and Jefferey L. **Burgess**. Pulmonary biomarkers based on alterations in protein expression following exposure to arsenic. Environ. Health Perspect. 115:585-591, 2007.  
DOI:10.1289/ehp.9611

**Burgess** Jefferey L., Mercedes M. Meza, Arun B. Josyula, Gerald S. Poplin, Michael J. Kopplin, H. E. McClellen, S. Sturup and R. Clark **Lantz**. Environmental arsenic exposure and urinary 8-OHdG in Arizona and Sonora. Clinical Toxicology 45:490-498, 2007.  
DOI: 10.1080/15563650701354119

Scott **Boitano**, Colin E. Olsen, A. Liguori, R. Clark **Lantz**. Environmental arsenic concentrations alter calcium signaling and wound repair in lung epithelial cells. Presented the Annual meeting of the Society of Toxicology, March 6, 2006, San Diego, CA

**Lantz**, R. Clark, Brandon J. Lynch, Scott **Boitano**, Gerald S. Poplin, Sally Littau , George Tsaprailis and Jefferey L. **Burgess**. Pulmonary biomarkers based on alterations in protein expression following exposure to arsenic. Presented at Experimental Biology 2006, April 3, 2006, San Francisco, CA

**Lantz**, R. Clark, Brandon J. Lynch, Scott **Boitano**, Gerald S. Poplin, Sally Littau , George Tsapralis and Jefferey L. **Burgess**. Pulmonary biomarkers based on alterations in protein expression following exposure to arsenic. USEPA Science Forum, May 17, 2006, Washington, DC.

“Role of Oxidative Stress in Arsenic-Induced Toxicity”, presented by R. Clark **Lantz** at the Biological Reactive Intermediates Symposium, January 4-7, 2006, Tucson, AZ.

“Pulmonary Toxicity of Arsenic” presented by R. Clark **Lantz** at the University of Pittsburgh, September 28, 2006.

“Health Impacts of Arsenic” presented by R. Clark **Lantz** at the "Symposium on Environmental Chemistry of Metal Pollution in the US-Mexico Border Region" at the 19th Rocky Mountain Regional Meeting of The American Chemical Society, October 16, 2006, Tucson, AZ

“Pulmonary Toxicity of Arsenic” presented by R. Clark **Lantz** at Sanofi/Aventis Pharmaceuticals, Oro Valley, AZ, June 6, 2007.

“Pulmonary Biomarkers Based On Alterations In Protein Expression Following Exposure To Arsenic” presented by R. Clark **Lantz** at the Annual meeting of the American Public Health Association, November 5, 2007, Washington, DC

Scott **Boitano**, A. E. Liquori, C. E. Olsen, Y. Zong, Jefferey L. **Burgess** and **R. Clark Lantz**. Low-dose arsenic inhibits wound repair, upregulates MMP-9 and alters  $\text{Ca}^{2+}$  signaling in human airway epithelial cells. Presented at Experimental Biology 2008, April 6, 2008, San Diego, CA.

(8) Annual Report Summary

(On subsequent pages).

## **NCER Assistance Agreement Annual Report Summary**

**Date of Report:** March 2, 2007

**EPA Agreement Number:** R832095

**Title:** Pulmonary Biomarkers Based on Alterations in Protein Expression Following Exposure to Arsenic

**Investigators:** R Clark Lantz, Scott A. Boitano and Jefferey L. Burgess

**Institution:** University of Arizona

**Research Category:** Environmental Health , Health Effects

**Project Period:** January 8, 2005 through January 7, 2009

**Objective of Research:** Exposure to arsenic (As) has been linked to lung cancer. Environmental exposure to these metals will result in multiple adverse effects, which can be characterized through evaluation of alterations in protein expression. We will evaluate such alterations as biomarkers of exposure and effect prior to the development of cancer. This study will use the technology of proteomics to evaluate and identify biomarkers of chronic environmental exposure to As by evaluating large numbers of proteins simultaneously. We will compare alterations in protein expression in exposed human populations in Arizona, human cell lines, and *in vivo* rodent studies. Patterns of alterations in protein expression will be identified. These will be correlated with alterations in DNA oxidation in induced sputum from the lung.

**Progress Summary/Accomplishments:** Our *in vitro* studies use human bronchial epithelial (16HBE14o-) cells. We have shown that arsenic delays the ability of cells to form monolayers and alters the ability of the cells to “repair” and artificial wound. Arsenic increased the levels of MMP-9 which may contribute to the alterations we have seen. Calcium signaling appears to also be affected by arsenic. Arsenic reduces the number of adjacent cells that respond to a “wound” by increasing their calcium levels. In addition, the time at which intracellular calcium is elevated after a “wound” is significantly reduced in arsenic exposed cells at concentrations as low as 60 ppb. Arsenic also altered functional tight junctions. Arsenic added at the time of plating greatly reduced the ability of the cells to form a functional monolayer. This was apparent even at 60 ppb. If cells were first allowed to form a tight monolayer prior to addition of arsenic, high levels of arsenic were still capable of reducing the epithelial resistance. Cells exposed under these conditions showed alterations in the levels and types of expression of tight junctional proteins, occludin and claudins. These new data further support altered wound healing and maintenance of a functional epithelial barrier as targets for arsenic exposure.

***In vivo* animal responses to arsenic exposure**

We have analyzed altered protein expression in the lung lining fluid and airway epithelial cells of mice exposed to arsenic in their drinking water for up to 4 weeks. Soluble proteins in the lung lining fluid were obtained through bronchoalveolar lavage (BAL). Proteins were identified using 2-D gel electrophoresis (N=3) or multidimensional protein identification technology (MUDPIT) (N=2) coupled with mass spectrometry (MS). A total of 44 proteins were identified. Proteins that were seen to be present in the BAL of control animals while absent in the treated animals include: glutathione-S-transferase omega-1 (GST-omega-1), contraspin, apolipoprotein A-I and A-IV, and receptor for advanced glycation end products (RAGE). Proteins up regulated by arsenic included enolase-1 and peroxiredoxin-6. Using Western Blot analysis, we have shown that levels of RAGE in the BAL decreases as a function of arsenic treatment in mice treated with arsenic in their drinking water. Previous investigators have identified GST omega-1 as an important arsenic metabolizing enzyme. The function of RAGE in chronic inflammatory disease, wound healing and cancer has been previously reported. In addition, alpha-1-antitrypsin levels were also affected by arsenic treatment. We have also used a selective airway epithelial cell digestion to isolate and analyze altered proteins. Using MUDPIT analysis we have identified over 150 proteins changed by arsenic. We have also analyzed the most likely cell functions and disease states that are associated with the altered proteins from BAL and from the cell fractions. Using a curated analysis system (MetaCore) we have identified cell motility and alteration in wound repair as the most likely affected cellular functions and disease. This is consistent with our *in vitro* data. In order to verify wound healing as a site of action of arsenic, we have examined the effect of arsenic on the time for recovery from epithelial damage caused by exposure to naphthalene. We have found that while normal recovery from naphthalene exposure occurs within 2 weeks of exposure, damage in animals that had been exposed to arsenic was still present after 2 weeks. These data indicate that arsenic can affect wound healing in an animal model.

### **Human population studies**

Based on the results from both the *in vitro* and *in vivo* experiments, we have analyzed arsenic-induced changes in MMP-9 and RAGE in induced sputum in human populations. Samples were collected from 56 individuals living in Ajo, Arizona (tap water arsenic ~ 20 ppb) and from Tucson, Arizona (tap water arsenic ~ 5 ppb). First morning void urine was also collected and arsenic speciation analysis was performed. Both MMP-9/TIMP1 ratio and RAGE were significantly altered as a function of arsenic intake. In this same population, we analyzed sputum alpha-antitrypsin (AAT) by ELISA, then performed multivariate analysis to determine which predictor variables were associated with AAT, both directly and corrected for total protein. In a regression model, total urinary inorganic arsenic was negatively associated with sputum AAT ( $p=0.028$ ) and AAT/protein ( $p=0.014$ ). Most recently, we have found that serum MMP-9 appears to be correlated with arsenic exposures. We are sampling additional individuals to verify this finding.

### **Publications/Presentations:**



Josyula, Arun B., Gerald S. Poplin, Margaret Kurzius-Spencer, Hannah E. McClellen, Michael J. Kopplin, Stefan Sturup, R. Clark **Lantz**, Jefferey L. **Burgess**  
Environmental arsenic exposure and sputum metalloproteinase concentrations.  
Environ. Research 102:283-290, 2006.

**Lantz**, R. Clark Lantz and Allison M. Hays. Role of oxidative stress in arsenic-induced toxicity. Drug Metabolism Reviews 38:791-804, 2006.

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**Burgess** Jefferey L., Mercedes M. Meza, Arun B. Josyula, Gerald S. Poplin, Michael J. Kopplin, H. E. McClellen, S. Sturup and R. Clark **Lantz**. Environmental arsenic exposure and urinary 8-OHdG in Arizona and Sonora. Clinical Toxicology 45:490-498, 2007.

**Future Activities:** For the *in vitro* experiments, we will continue to investigate calcium signaling as a site of action. For *in vivo*, we will use quantitative morphological techniques to verify delays in wound healing in the distal lung of animals exposed to arsenic. We will continue to analyze our proteins identified as potential biomarkers. Finally, our human studies will also continue, with the inclusion of samples collected from a Mexican population that is being exposed to 50 ppb and higher arsenic levels in their drinking water. This will broaden our current range of exposures to give us more confidence in the dose-responses we are seeing. We will also test alterations of MMP-9 in serum as a biomarker of arsenic exposure and effect.

**Supplemental Keywords:** heavy metals, human health, animal, cellular, biomarkers, induced sputum, bronchial epithelial cells , Health, PHYSICAL ASPECTS, RFA, Scientific Discipline, Water, Arsenic, Biochemistry, Environmental Chemistry, Hazardous Waste, Health Risk Assessment, Physical Processes, Risk Assessments, arsenic exposure, bioaccumulation, biogeochemistry, biomarker measurements, contaminant transport, contaminated sediments, exposure

**Relevant Web Sites:** none